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Transgene expression for *Gladiolus* plants grown outdoors and in the greenhouse

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ABSTRACT

Transgene expression was evaluated for *Gladiolus* plants transformed with either the CaMV 35S, double CaMV 35S, *rolD*, or *Arabidopsis UBQ3* promoter controlling the *uidA* or bean yellow mosaic virus coat protein gene in either the sense or antisense orientation to determine differences in expression for plants grown in the greenhouse and outdoors for two years. There was more variability in GUS expression when plants were grown outdoors than in the greenhouse for two years. Four of the six transformed plant lines with the *UBQ3*, *rolD*, and CaMV 35S promoters grown outdoors showed significant differences in GUS expression from year to year as compared to two of the six lines with the *UBQ3* and *rolD* promoters grown in the greenhouse. When grown the same year, two plant lines with the CaMV 35S and one line with the *rolD* promoter showed 2–16× higher levels of GUS expression outdoors than in the greenhouse, and one plant line with the *UBQ3* promoter had 31× higher GUS expression in the greenhouse instead of outdoors. Three of six plant lines transformed with the bean yellow mosaic virus coat protein gene in either the sense or antisense orientation under control of the double CaMV 35S promoter showed obvious transgene expression as compared to three lines that did not show expression or negligible expression for both years when plants were grown both outdoors and in the greenhouse. This study verified long-term gene expression, rather than silencing, for *Gladiolus* plants when grown outdoors and in the greenhouse from year to year.

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1. Introduction

Gladiolus is an important cutflower and garden flower that can benefit from genetic engineering for virus and fungus resistance. Most of our transgenic *Gladiolus* plants have been grown in the greenhouse under federal guidelines for containment of genetically engineered plants, but ultimately transgenic *Gladiolus* plants will be grown outdoors as they are not traditionally grown in the greenhouse. Transgenic plants grown and found to be resistant to a pathogen in the greenhouse are frequently not resistant in the field (Anand et al., 2003). It is not known if the difference in resistance is due to changes in transgene expression that occur when plants are grown outdoors resulting in less resistance to the pathogen, if there is a higher inoculum of pathogen outdoors, or other uncharacterized factors.

The environment where a plant is grown has been found to affect transgene expression in some cases. Others have found changes in transgene expression that occur when their plants are grown outdoors. Petunias transformed with the maize *A1* gene under control of the CaMV 35S promoter exhibited the expected salmon red flowers when plants were grown in the field, however, some flowers were white or a lighter red color (Linn et al., 1990; Meyer et al., 1992). Only 5% of the petunias grown in the greenhouse had light red flowers as compared to over 60% of the field-grown petunias with light red flowers. The white and lighter red flowers were found to have a hypermethylated CaMV 35S promoter that was attributed to both the environmental conditions in the field and the age of the parental plant when they were pollinated. The exact environmental conditions in the field that resulted in methylation of the CaMV 35S promoter are unknown.

An elevated temperature, 30–37 °C, has been shown to reduce expression of some transgenes. Expression of the luciferase gene under control of the CaMV 35S promoter and of the neomycin phosphotransferase gene under control of the nopaline synthase promoter was reduced in some *Nicotiana tabacum* plant lines (Neumann et al., 1996; Connor et al., 1998). This reduced expression of the transgene could not be explained by changes in methylation (Neumann et al., 1996). Silencing of the *nptII* gene in *Arabidopsis thaliana* plants occurred in 11 of 16 lines when the plants were grown at 30 °C (Meza et al., 2001). *Medicago sativa* suspension cells transformed with the phosphinothricin-*N*-acetyltransferase gene under control of the CaMV 35S promoter became susceptible to phosphinothricin when cells were cultured at 37 °C (Walter et al., 1992). An aleurone-specific gene, *Ltp2-gus*, was silenced more frequently when plants were grown in the

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Abbreviations: 2,4-D, 2,4-dichlorophenoxyacetic acid; BYMV, bean yellow mosaic virus; CP, coat protein; GUS, β-glucuronidase.

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greenhouse during the summer (25–33 °C) than winter (19–25 °C) (Morino et al., 1999). In comparison, two transgenic lines of barley with the *bar* and *uidA* genes under control of the *Ubi* promoter continued to express both *bar* and GUS when plants were exposed to either water and nutrient deprivation or heat shock at 31 °C (Meng et al., 2006).

Growing transgenic plants in vitro prior to their transfer to the greenhouse or field can cause silencing as observed with *N. tabacum* plants transformed with either the nitrate reductase or nitrite reductase genes under control of the CaMV 35S promoter (Palauqui et al., 1996). A high frequency of GUS and *bar* silencing occurred when barley plants of a particular line were cultured in vitro (Meng et al., 2006).

Previous studies with transgenic *Gladiolus* plants and cell lines have characterized *Gladiolus* as being a plant that, unlike many other plants, generally expresses transgenes despite having multiple, rearranged copies of the transgene. Eight of 11 *Gladiolus* plants transformed with the BYMV coat protein gene in either the sense or antisense orientation showed transcription of the transgene as determined by Northern hybridization (Kamo et al., 2005). Over 500 *Gladiolus* cell lines were isolated, and 72% of the cell lines with the *bar-uidA* fusion gene expressed GUS. Only 5% of these cell lines expressing GUS no longer expressed GUS after one year in vitro (Kamo et al., 2000). Long-term GUS expression continued for all 23 *Gladiolus* plants grown in vitro and in the greenhouse after three seasons of dormancy, but this is the first study showing continued expression outdoors of those lines (Kamo, 2003).

In this study transgenic *Gladiolus* plants expressing three different transgenes under the control of four different promoters were studied to determine if transgene expression would change significantly once the greenhouse-grown plants were grown outdoors. Transgenic plant lines expressing varying levels of the transgene were included to examine reduction or an increase in expression.

2. Materials and methods

2.1. Transformation

Callus was induced from in vitro-grown plants or cormel slices of *Gladiolus* cv. Jenny Lee on MS basal salts medium (Murashige and Skoog, 1962) containing 3% (w/v) sucrose, 2.0 mg l^{-1} glycine, 1.0 mg l^{-1} thiamine, 0.5 mg l^{-1} pyridoxine, 0.5 mg l^{-1} nicotinic acid, either 2.3 μM 2,4-D or 9.0 μM dicamba (3,6-dichloro-2-methoxybenzoic acid) and 0.2% Phytagel (Sigma, St Louis, MO), pH 5.8 (Kamo et al., 1995). Suspension cells were initiated from the callus and used for gene gun bombardment using the same medium as for callus but without the Phytagel. Callus and suspension cells were maintained in the dark at 26 °C. Suspension cells were grown on a gyratory shaker at 100 rpm and half of the cells in a flask were transferred every two weeks to 30 ml fresh medium. Callus was transferred monthly to fresh medium.

The gene gun was used for delivery of DNA (Kamo, 2003; Kamo et al., 2005). Plasmid DNA was isolated by alkaline lysis followed by purification on a cesium chloride gradient (Maniatis et al., 1982). DNA was delivered to the suspension cells using the PDS-1000/He system (BioRad, Richmond, CA). Gold or M10 tungsten particles (1.0 μm) were coated with DNA according to Sanford et al. (1993). Suspension cells were used to cover a 55 mm diameter Whatman no. 4 filter paper that was then bombarded once per Petri plate at 8.3 MPa (1200 psi) using a target distance of 12 cm. The gene gun was set with a 1 cm gap and 1 cm flying membrane distance.

Three of the plasmids for bombardment consisted of the *bar-uidA* fusion gene under control of either the CaMV 35S (pDM327: D. McElroy, Maxygen, Redwood, CA), *rolD* (psan 80: F. Leach, Center of Versailles Laboratorie de Biologie de la Phizosphere, Cedex, France) (Leach and Ayogi, 1991), or *Arabidopsis UBQ3* (psan 152: J. Callis, University of California, Davis, CA) (Norris et al., 1993) promoter (Fig. 1). Two of the plasmids had the BYMV coat protein gene in either the sense or antisense orientation under control of the double CaMV 35S promoter (pOCP and pAS: J. Hammond, USDA, Beltsville, MD) (Kamo et al., 2005). Cells were co-bombarded with the coat protein gene and p35SAc that contains the phosphinothricin acetyltransferase gene under control of the CaMV 35S promoter (received from AgrEvo, Somerville, NY) for selection using *pat* as the selectable marker gene.

Bombarded cells were transferred one week after shooting to MS basal salts medium supplemented with 2.3 μM 2,4-D and with either bialaphos (1–3 mg l^{-1}, www.meiji.co.jp) or phosphinothricin (6 mg l^{-1}, AgrEvo, Somerville, NJ) for selection of the putatively transformed callus. Transfers were made monthly to fresh medium for 3–6 months. Callus was then grown under a 16/8 h (light/dark photoperiod) on MS basal salts medium supplemented with 9.3 μM kinetin and either 1 or 3 mg l^{-1} bialaphos for regeneration of plants. Regenerated plants were grown in Magenta jars on MS basal medium without hormones and without a selection agent.

2.2. Transgenic plants

Transgenic *Gladiolus* plants with the *uidA* gene under control of the CaMV 35S, *rolD*, and *UBQ3* promoters were selected from a previous study where they expressed relatively moderate levels of GUS expression (Kamo, 2003). GUS expression was similar for plants with the CaMV 35S and *UBQ3* promoters grown in vitro and in the greenhouse, but expression was typically higher for *rolD*-containing plants grown in the greenhouse as compared to in vitro. Transgenic *Gladiolus* plants with the BYMV coat protein (CP) gene in the sense and antisense orientations were chosen based upon previous transgene expression results (Kamo et al., 2005). Both a

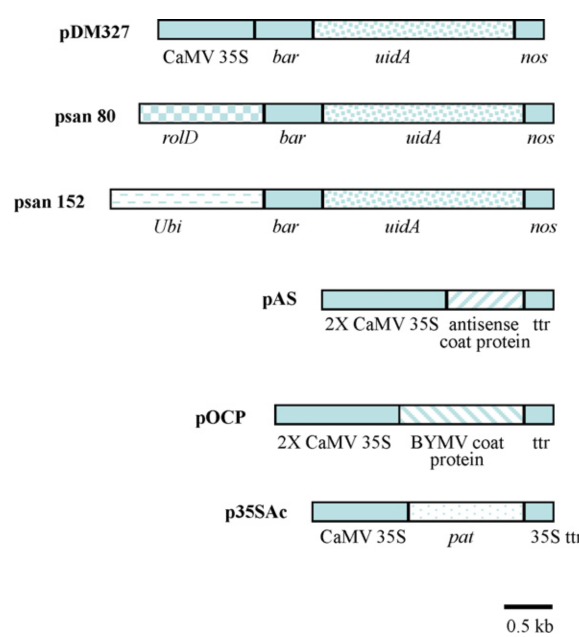

Fig. 1. Plasmid DNA constructs used for transformation experiments. From top to bottom: *bar-uidA* fusion gene under control of the 35S RNA promoter from the cauliflower mosaic virus (pDM327), *Agrobacterium tumefaciens rolD* promoter (psan 80), and *Arabidopsis UBQ3* ubiquitin promoter (psan 152), antisense orientation of the BYMV coat protein gene (pAS) under control of the double 35S RNA promoter, the BYMV coat protein gene (pOCP) under control of the double 35S RNA promoter, and the *pat* gene (p35SAc) under control of the 35S RNA promoter.

line that had expressed high levels of RNA (P-184 for antisense and P-183 for the sense orientation of BYMV coat protein) and low levels (P-185 for antisense and P-238) were included in this study. All transgenic plants had a relatively low copy number for gene gun bombardment. Most plants had 2–6 copies of the transgene, and one plant had 8 copies.

Transgenic plants had been grown for at least three seasons in the greenhouse before being used for this study. Many plants had flowered during this time. Progeny could not be obtained by self-pollinating, sib pollinating, or cross-pollinating with non-transgenic plants of other cultivars.

2.3. Plant growth

Plants were grown in the greenhouse from April through November by planting corms that had been stored at 4 °C. The greenhouse was maintained at 24–25 °C/21–23 °C (day/night).

Plants were grown indoors and outdoors in clay pots filled with MetroMix 200 (Scotts Company, Marysville, OH). Plants were grown outdoors in 2003 and 2005 under APHIS permit numbers 03-059-01n and 01-130-01n, respectively. Osmocote 14-14-14 fertilizer was added to each pot at the time of planting. Throughout the growing season, plants were treated with Marathon, 1% granular (Olympic Horticultural Products, Co., Mainland, PA), Safer Brand Insecticidal Soap (Ringer Corp., Bloomington, MN), Avid, 0.15 EC (Novartis Crop Protection, Inc., Greensboro, NC), Concern Insect Killing Soap (Woodstream Corp., Lititz, PA), Thuricide (Bonide Products, Inc., Oriskany, NY), Bonide Insecticidal Soap (Bonide Products, Inc.), and Knock-Out Gnats (Gardens Alive!, Inc., Lawrenceburg, IN) as needed.

Young, newly-developed leaves, the fourth leaf to emerge, were generally collected for analysis of transgene expression and stored at −70 °C until analyzed for either GUS activity or RNA levels.

2.4. GUS activity

Leaf tissue was collected from the basal section of the fourth young leaf for determination of the specific activity of GUS expression according to Jefferson et al. (1987). Approximately 300 mg fresh weight of leaf tissue was ground in 500 μl extraction buffer (50 mM NaH_2PO_4, pH 7.0, 10 mM EDTA, pH 8.0, 0.1% Triton X-100, 0.1% sarkosyl, 10 mM β-mercaptoethanol) using Lysing Matrix tubes with two spheres/tube (Bio101 Fast DNA Kit, MP Biomedicals, Irvine, CA) shaken in the Fast Prep FP120 instrument (Qbiogene, Inc., Carlsbad, CA). The supernatant was collected following centrifugation at 16,000 g at 4 °C in a microcentrifuge, and an aliquot of the supernatant was added to the assay buffer (1 mM methyl umbelliferyl-β-D-glucuronide). The samples in assay buffer were incubated at 37 °C, and aliquots were added to 0.2 M sodium carbonate after 0, 15, 30, and 60 min incubation. Fluorescence was measured with a BioRad VersaFluor Fluorometer set at 360/40 nm for excitation and 460/10 nm for emission. Protein concentration of the tissue extracts was determined using the bicinchoninic (BCA) protein assay reagent (Pierce, Rockford, IL) according to the manufacturer's instructions.

2.5. RNA analysis

RNA was isolated using lithium chloride (Vervoerd et al., 1989) and then used for electrophoresis in a MOPS/formaldehyde gel. For samples that showed degradation of the RNA, the RNA was re-isolated using the RNeasy Kit (Qiagen,Valencia, CA). Ethidium bromide was added to the RNA samples when loaded into the wells of the gel to visualize the quality and relative amounts of RNA loaded on each gel. Each lane contained 30 μg of RNA. RNA was transferred by capillary movement to a Nytran membrane. RNA blots were incubated in Ultrahyb buffer (Ambion, Austin, TX) for 1 h at 42 °C followed by 16 h hybridization at 42 °C with labeled probe in Ultrahyb buffer.

Probes were isolated by gel purification using Prepagene (BioRad, Richmond, CA) according to the manufacturer's instructions and then labeled with α-[^{32}P]dCTP using the DECAprime II kit (Ambion). The probe for the plants transformed with the BYMV CP gene was a 1.05 kb insert of the CP gene. Pst I digestion of plasmid p49Y6 that contains the CP gene but lacks both a promoter and transcription termination region released the 1.05 kb insert of the CP gene used as the probe (Hammond and Hammond, 1989). The probe for the plants carrying the CP gene in the antisense orientation was a 660 bp insert of the CP antisense gene. Plasmid pBY6 that contains the CP gene in the antisense orientation and lacking both a promoter and translation termination region was digested with Pst I to release a 660 bp insert of the CP antisense gene used as the probe. Following hybridization blots were washed at 42 °C for 15 min each wash, in 2× SSC, 0.2% SDS, then 1× SSC, 0.2% SDS, and lastly in 0.1× SSC, 0.2% SDS before exposing the blot to X-ray film at −70 °C for 1–5 days.

2.6. Statistical analysis

Each year 3–4 different plants for each independently transformed plant line were analyzed for determining the specific activity of GUS expression or comparing levels of RNA transcription by Northern hybridization. A *t*-test was performed on the mean specific GUS activity comparing plants grown either in the greenhouse or outdoors from year 1 and year 2 to determine if GUS expression was significantly different between years at $P \leq 0.05$. A *t*-test was performed on the mean specific GUS activity comparing plants grown in the greenhouse or outdoors during either year 1 or year 2 to determine if GUS expression was different.

3. Results and discussion

3.1. GUS expression

All transgenic plant lines used in this study were previously characterized by Southern hybridization (Kamo, 2003). Plant lines CaMV 35S P-2 and *rolD* P-14 contained a single copy of the transgene whereas the other plant lines contained 2–6 copies of the transgene. Every plant derived from the original, transformed plant was not confirmed by PCR or Southern hybridization to contain the transgene, but it was assumed that the transgenic plants selected for this study were not chimeric because regeneration has been shown to occur from embryogenic callus (Kamo et al., 1990). Multiple plants that regenerated from a single, transformed piece of callus have each been confirmed by PCR to contain the transgene in a previous study (Kamo et al., 1995).

Four plant lines, *UBQ3* P-9, CaMV 35S P-2, CaMV 35S P-32, and *rolD* P-32 showed significant differences in GUS expression between greenhouse and outdoor-grown plants during the same year (Table 1). The levels of GUS expression were 2-16× higher outdoors than in the greenhouse for plant lines CaMV 35S P-2, CaMV 35S P-32, and *rolD* P-32, respectively. This was unexpected because silencing of the CaMV 35S promoter has occurred for petunia plants, both the primary transformants and their progeny, grown outdoors in the field. The change in maize *A1* gene expression seemed to be affected by both the environmental conditions in the field as well as age of the parental plant at the time plants were pollinated for seeds (Meyer et al., 1992).

Table 1
Specific activity of GUS expression for *Gladiolus* plants containing the *uidA* gene under control of either the CaMV 35S, *Arabidopsis UBQ3*, or *rolD* promoters

Promoter	Plant line	Location	Year 1 (nmol 4-MU/min/mg)	Year 2 (nmol 4-MU/min/mg)
NT		GH	23.7	21.8
NT		Outdoors	22.4	22.4
UBQ3	P-4	GH	539.2	814.3
UBQ3	P-4	Outdoors	513.9 a	856.9 b
UBQ3	P-9	GH	63.3 c	796.1* d
UBQ3	P-9	Outdoors	19.8	26.3*
CaMV 35S	P-2	GH	2836.7	7558.3*
CaMV 35S	P-2	Outdoors	2206.2 e	18205.1* f
CaMV 35S	P-32	GH	96.6*	49.3
CaMV 35S	P-32	Outdoors	1527.2* g	258.0 h
rolD	P-14	GH	239.0	138.6
rolD	P-14	Outdoors	308.9	237.6
rolD	P-32	GH	91.7 i	24.8* j
rolD	P-32	Outdoors	71.4 k	152.8* l

Plants were grown either in the field or greenhouse for two years. Leaves from 3–4 transformed plants/line and non-transformed (NT) plants were analyzed. Values shown are the mean specific activity and differences in expression are indicated according to the *t*-test at $P \leq 0.05$. Different letters (a–l) indicate that the expression was significantly different for the same plant line grown either in the greenhouse or outside each year.
* GUS expression was significantly different between greenhouse and outdoor-grown plants.

There was more variation in GUS expression when plants were grown outdoors than in the greenhouse, between the two years. Four of the six plant lines, *UBQ3* P-4, CaMV 35S P-2, and *rolD* P-32 showed 2–8× higher levels of GUS expression the second year outdoors as compared to the first year, and one line, CaMV 35S P-32 showed a 5× lower level of expression the second year grown outdoors. The upper temperature limit for the greenhouse and outdoors was similar, but the low temperature for outdoors was always considerably lower than that in the greenhouse. In July, August, September and October of 2005, the high/low temperatures for the greenhouse were 36/22, 36/22, 34/19, 28/19 and outdoors was 39/17, 38/14, 34/12, and 35/6 °C, respectively. Possibly the wider range in temperature that occurred outdoors daily resulted in more variation in GUS expression for outdoor-grown plants.

This study showed that although there was more variation in GUS expression for lines grown outdoors as compared to in the greenhouse, GUS expression continued for plants grown outdoors even when the *uidA* gene was under control of the CaMV 35S promoter. The only exception was *UBQ3* P-9 that had consistently high levels of GUS expression (3–36× that of non-transformed plants) when grown in the greenhouse but no detectable GUS expression in the leaves when grown both years outdoors.

3.2. Bean yellow mosaic virus coat protein and antisense coat protein expression

Plant lines with the BYMV coat protein gene in the antisense orientation under control of the double CaMV 35S promoter, P-184, P-185, and P-415, contained 2, 8, and 6 copies, respectively, of the transgene (Kamo et al., 2005). Degradation of the RNA was an occasional problem when RNA was isolated from plants growing in both the greenhouse and outdoors although two different methods of RNA extraction were used (Figs. 2 and 3). When one considers only those plants with decent, undegraded RNA extracted from them, plant line P-184 produced RNA transcripts that hybridized to the antisense probe for all six plants grown in the greenhouse and for four of four plants grown outdoors (Fig. 2). Line P-415 showed only a very low level of RNA antisense expression for two of five plants grown outdoors, and none of the five plants grown in the greenhouse showed antisense expression. Line P-185 appeared to be silenced because there was no hybridization with the RNA antisense probe for the five plants grown in the greenhouse and the five plants grown outdoors. Northern hybridization showed obvious antisense RNA expression occurred for line P-184, and it was either absent or negligible for lines P-415 and P-185 when plants were grown either in the greenhouse or outdoors for two years.

Three plant lines containing the BYMV coat protein gene in the sense orientation under control of the double CaMV 35S promoter, P-183, P-232, and P-238, contained 2, 2, and 3 copies of the transgene, respectively, as verified by Southern hybridization (Kamo et al., 2005). Coat protein was expressed by lines P-183 and P-238 when plants were grown in the greenhouse or outdoors (Fig. 3). Nine of 12 plants for line P-183, and seven of nine plants for line P-238 showed obvious coat protein expression when plants were grown in the greenhouse or outdoors for two years. In comparison, none of the 10 plants for P-232 showed coat protein expression both years when grown in the greenhouse or outdoors. This comparison of BYMV antisense and coat protein expression, or lack of expression by coat protein line P-232 and antisense line P-185, continued for two seasons and was not influenced by growth in the greenhouse or outdoors.

In conclusion, transgene expression continued during two seasons for *Gladiolus* plants grown in the greenhouse and outdoors. Silencing of previously-reported expressing lines was not observed during two years of growth in the greenhouse and outdoors. Transgene expression was higher in 3 out of 12 plant lines grown outdoors as compared to 1 out of 12 plant lines grown indoors. *Gladiolus* plants are always grown outdoors for cutflower and garden use. They are never grown in the greenhouse probably because it is much more difficult to grow them in the greenhouse where they easily rot and become rapidly infested with spider mites. The higher levels of transgene expression for *Gladiolus* plants grown outdoors may sometimes be because the plants grown outdoors were healthier, growing more vigorously, producing more RNA and proteins than the plants in the greenhouse, but this does not explain why UBQ3 P-9 consistently expressed GUS as higher levels when grown in the greenhouse rather than outdoors. There are undoubtedly other factors each contributing differently to transgene expression for each transformed line such as location in the genome of each transgene that may affect endogenous genes and their expression.

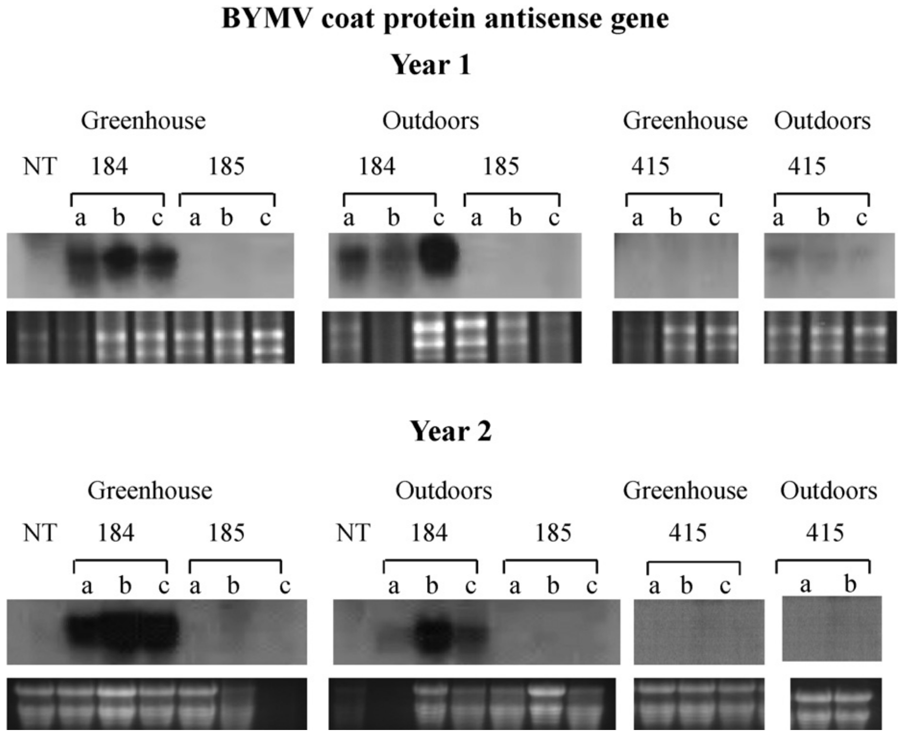

Fig. 2. RNA transcripts shown for 2–3 plants (a–c) of independently transformed *Gladiolus* plant lines, P-184, P-185, P-415, containing the BYMV coat protein gene in the antisense orientation under control of the double CaMV 35S promoter. NT RNA is from a non-transformed plant line. Plants were grown for two years either in the greenhouse or outdoors. Each lane contained approximately 30 μg RNA visualized by ethidium bromide staining (bottom lanes). The RNA was hybridized with a radioactively-labeled probe (top lanes).

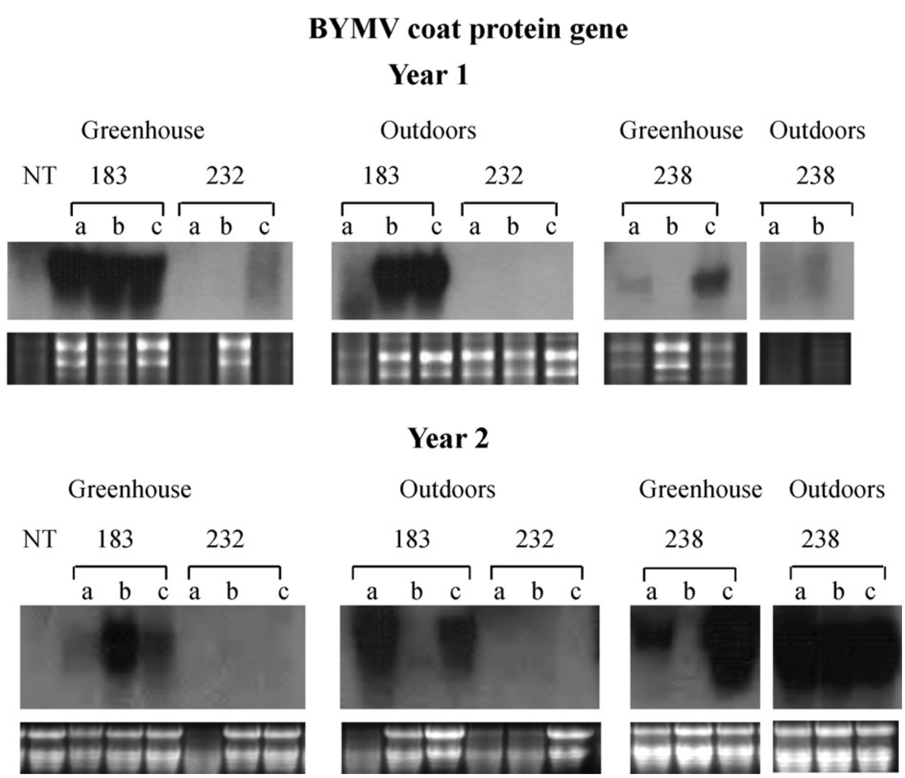

Fig. 3. RNA transcripts shown for 2–3 plants (a–c) of independently transformed *Gladiolus* plant lines, P-183, P-232, P-238, containing the BYMV coat protein gene in the sense orientation under control of the double CaMV 35S promoter. NT RNA is from a non-transformed plant line. Plants were grown for two years either in the greenhouse or outdoors. Each lane contained approximately 30 μg RNA visualized by ethidium bromide staining (bottom lanes). The RNA was hybridized with a radioactively-labeled probe (top lanes).

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